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Biochimica et Biophysica Acta 1314 (1996) 167–174

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

# Tyrosine phosphorylation and translocation of phospholipase C-γ 2 in polymorphonuclear leukocytes treated with pervanadate

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Received 3 June 1996; accepted 7 June 1996

**Abstract**

We examined in detail the tyrosine phosphorylation of proteins, especially inositol phospholipid-specific phospholipase C (PLC) γ 2, during activation of respiratory burst of guinea pig polymorphonuclear leukocytes (PMNs) by pervanadate. The pervanadate, generated from a combination of $H_2O_2$ and orthovanadate, induced concomitantly tyrosine phosphorylation of 145, 120, 104, 76, 68, 60, 53, 42, 37, 28, and 25 kDa proteins and superoxide anion ($O_2^-$) production of PMNs. The pretreatment of PMNs with genistein caused an inhibition of tyrosine phosphorylation of these proteins, and also markedly depressed $O_2^-$ production. Among the above proteins, a 145 kDa protein was found to be identical with the protein recognized by the anti-PLCγ 2 antibody on Western blots. PLCγ 2 was detected in the cytosol fraction but not in the membrane fraction of resting PMNs, whereas it was detected in both cytosol and membrane fractions of pervanadate treated PMNs. PLC activity of pervanadate treated PMNs was higher than that of resting cells. In addition, the enzyme activity of the cytosol fraction from the former cells was significantly lower than that from the latter cells, whereas the enzyme activity of membrane fraction from the former cells was significantly higher than that from the latter cells. These findings suggest that the tyrosine residue(s) of PLCγ 2 is phosphorylated and the enzyme is translocated from the cytosol to membrane fractions in PMNs by pervanadate treatment.

*Keywords:* Leukocyte; Tyrosine-phosphorylated protein; Phospholipase C; NADPH oxidase; Pervanadate; Translocation

## 1. Introduction

Polymorphonuclear leukocytes (PMNs) play an important role in host defence against microbial infections. Exposure of PMNs to a variety of stimuli results in activation of the respiratory burst, during which reactive species such as superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) are generated [1,2]. These active oxygen species play an important role in the bactericidal activity of PMNs [3]. The signaling pathway through the activation of inositol phospholipid-specific phospholipase C (PLC) plays a major role in activation of NADPH oxidase that exists in the plasma membrane and is able to catalyze the

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*PII* S0167-4889(96)00077-8

one-electron reduction of molecular oxygen to $O_2^-$ during the respiratory burst. That is, the binding of agonist to their receptors stimulates PLC through the activation of guanine nucleotide binding proteins (G-proteins) [4]. Diacylglycerol produced by PLC activates NADPH oxidase presumably through activating protein kinase C (PKC) [5].

Recently, Bianchini et al. reported that in HL 60 cells, vanadates, a tyrosine phosphatase inhibitor, in the presence of $H_2O_2$ promote the PLCγ 2-mediated production of inositol 1,4,5-trisphosphate ($IP_3$) through tyrosine phosphorylation of PLCγ 2 [6]. In addition, Dusi et al. reported that in human neutrophils Fcγ receptor activation by insoluble immune complex caused a stimulation of phosphoinositide hydrolysis and upon Fcγ receptor activation tyrosine phosphorylation of PLCγ 2 with a molecular mass of 145 kDa occurred [7]. However, the function of the phosphorylated PLCγ 2 in NADPH oxidase activation is yet obscure.

Herein, we examined in detail the tyrosine phosphorylation of PLCγ 2 during the activation of respiratory burst of guinea pig PMNs. We used pervanadate generated by treatment of orthovanadate with $H_2O_2$, since it has been shown to enhance the accumulation of phosphotyrosine-containing proteins in T lymphocytes [8] and RBL-2H3 cells [9]. We show here that PLCγ 2 is expressed by guinea pig PMNs and is present in the cytosol of the resting cells but not in the membrane, and that treatment of PMNs with pervanadate results in tyrosine phosphorylation of PLCγ 2, and in translocation of the phosphorylated PLCγ 2 to membrane fractions from the cytosol.

## 2. Materials and methods

### 2.1. Materials

L-α-[*myo*-inositol 2-$^3$H(N)]Phosphatidylinositol (1.0 Ci/mol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA) Phosphatidylinositol (soybean, ammonium salts), phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), benzamidine, cytochrome *c* (type III), catalase, sodium orthovanadate were from Sigma (St. Louis, MO, USA), $H_2O_2$ from Wako Pure Chemicals (Osaka, Japan), and genistein was from Dojindo (Kumamoto, Japan). Anti-phosphotyrosine mouse monoclonal antibody, and anti-PLCγ 1 and -PLCγ 2 rabbit polyclonal antibodies were purchased from Transduction Laboratories (KY, USA) and Santa Cruz Biotechnology (CA, USA), respectively. All other chemicals used were of reagent grade and were obtained commercially.

### 2.2. Preparation of PMNs

Guinea pig PMNs were obtained from casein-induced peritoneal exudates from female Hartley strain guinea pigs as described previously [10].

### 2.3. Preparation of pervanadate

One ml of 10 mM sodium orthovanadate and 1 ml of 0.3 M $H_2O_2$ was mixed and incubated in Krebs-Ringer's phosphate buffer (pH 7.0) (KRP) for 1 min at 37°C, and then 0.5 ml of catalase solution (2 mg/ml) was added to decompose the remaining $H_2O_2$. The solution thus obtained was used as 4 mM pervanadate, unless otherwise stated. This solution was newly prepared in each case.

### 2.4. Stimulation of PMNs with pervanadate

PMNs ($10^7$ cells/ml) were incubated in the presence and absence of pervanadate in KRP at 37°C with shaking. In the experiment with genistein, PMNs ($10^7$ cells/ml) were pretreated with genistein (100 μM) for 5 min at 37°C before the incubation with pervanadate. After incubation with pervanadate for the duration indicated, cell suspensions were withdrawn and analyzed for $O_2^-$ production, PLC activity, and immunochemical detection of phosphotyrosine proteins and PLCγ family.

### 2.5. Preparation of cytosol and membrane fractions

After treatment of PMNs with pervanadate for 3 min, cells were separated by centrifugation (100 × *g*, 5 min at 4°C) and were resuspended in ice-cold extraction buffer (10 mM Hepes, pH 7.0, containing 0.32 M sucrose, 0.05% $NaN_3$, 100 μM $Na_3VO_4$, 3 mM EGTA, 5 mM benzamidine, 0.1 mM PMSF, 0.5 mM DFP, and 5 mM 2-mercaptoethanol). After soni-

cation of the cell suspension, the supernatant and precipitation were separated by centrifugation (85 000 × *g*, 30 min at 4°C), and the precipitation was resuspended in extraction buffer. The supernatant and precipitation thus obtained were used as cytosol and membrane fractions, respectively.

### 2.6. $O_2^-$ determination

$O_2^-$ production by PMNs was measured on the basis of the reduction of ferricytochrome *c* by the anion produced. In brief, PMN samples ($10^6$ cells/ml) were incubated with cytochrome *c* (50 μM) and glucose (5 mM) in KRP with shaking. After appropriate incubation, superoxide dismutase (10 μg/ml) was added to stop the reaction, and then the supernatant was separated by centrifugation. Reduced cytochrome *c* in the supernatant was measured on the basis of increase in absorbance at 550 nm, and $O_2^-$ generation was calculated by using an absorption coefficient of 19.1 $mM^{-1}$ $cm^{-1}$ at 550 nm [11] for the reduction of cytochrome *c*.

### 2.7. PLC activity

The PLC activity was measured according to the method described previously by using phosphatidylinositol as a substrate [12]. In brief, the reaction mixtures contained 280 μM phosphatidylinositol, 30,000 dpm of L-α-[*myo*-inositol 2-$^3$H(N)]phosphatidylinositol, 1 mg/ml sodium deoxycholate, 1.5 mM $CaCl_2$, 50 mM Hepes (pH 7.0), and enzyme fractions. After incubation at 37°C, the reaction was stopped with 1 ml of chloroform/methanol/concentrated HCl (50:50:0.3), followed by the addition of 0.3 ml of 1M HCl containing 5 mM EGTA. After centrifugation for 10 min at 3000 × *g*, a 700-μl aliquot of the supernatant was removed for liquid scintilation counting. PLC activity was defined as nmol of inositolphosphate released from phosphatidylinositol. The protein concentration was measured by the method of Lowry et al. [13].

### 2.8. Immunochemical detection of phosphotyrosine proteins and the PLCγ family

Each cell suspension, whole extract, cytosol fraction or membrane fraction, was mixed 1:1 with lysis buffer (2% sodium dodecyl sulfate (SDS), 30% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue dye in 0.25 M Tris-HCl, pH 6.8) [14] and were heated at 100°C for 3 min, and then were subjected to electrophoresis on 4–20% SDS-polyacrylamide gels, electrotransferred to Immobilon (Millipore), blocked for 1 h with phosphate-buffered saline containing 0.1% Tween 20 (TPBS), and then incubated with an anti-phosphotyrosine mouse monoclonal antibody (1:500) or anti-PLCγ 1 or -PLCγ 2 rabbit polyclonal antibodies (1:500) in PBS containing 3% BSA for 18 h at 4°C. Blots were washed with TPBS, incubated with biotinylated anti-mouse or anti-rabbit IgG (Vector, CA, USA) in PBS, washed again with Tris-buffered saline, and then visualized with diaminobenzidine and $H_2O_2$. For the immunoprecipitation, cytosol fractions obtained from resting and pervanadate treated PMNs as described above were immunoprecipitated with anti-phosphotyrosine antibody conjugated with agarose (Transduction Laboratories). The precipitates collected by brief centrifugation were subjected to electrophoresis and then immunoblotting by using anti-phosphotyrosine antibody or anti-PLCγ 2 antibody as described above.

### 2.9. Statistical evaluation

The results are given as mean ± standard error (S.E.) values. Statistical significance was determined using ANOVA and Dunnett's two-tailed test. Analysis was performed using STATVIEW II software (Abcus Concepts) and a Macintosh personal computer (Apple Computer).

## 3. Results

### 3.1. $O_2^-$ production and tyrosine phosphorylation of proteins in pervanadate treated PMNs

In guinea pig PMNs incubated with pervanadate, $O_2^-$ production observed was dependent on the reaction time, especially, $O_2^-$ production was rapidly increased at the incubation step from 2 to 3 min (Fig. 1A). The incubation of PMNs with pervanadate resulted in the tyrosine phosphorylation of 145 kDa protein and other many proteins including at least 120, 104, 76, 68, 60, 53, 42, 37, 28, and 25 kDa

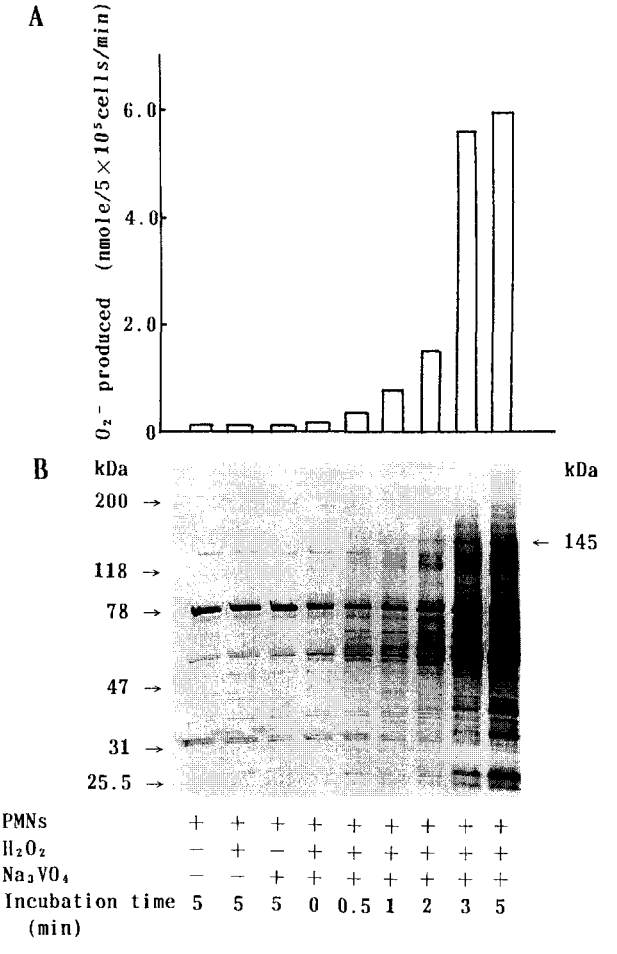


Fig. 1. $O_2^-$ production and phosphotyrosine proteins of PMNs treated with pervanadate. To prepare the vanadate or pervanadate solution for incubation with PMNs, vanadate alone, $H_2O_2$ alone or the combination of vanadate and $H_2O_2$ was incubated with KRP at 37°C for 1 min, and then catalase was added to the incubated solution as described in Section 2. PMNs ($10^7$ cells/ml) were incubated at 37°C for the indicated times, and then the cell suspensions were withdrawn and analyzed for $O_2^-$ production (A) and phosphotyrosine proteins (B) as described in Section 2.

proteins on the step inducing more rapid $O_2^-$ release (Fig. 1B). To know the relationship between $O_2^-$ release and tyrosine phosphorylation of these proteins, we studied the effect of genistein, a tyrosine kinase inhibitor. As shown in Fig. 2A, $O_2^-$ production was depressed to approximately 40% by the pretreatment of PMNs with genistein (100 μM). Gesistein was not toxic to PMNs in this experiment as the cell viability estimated by trypan blue staining was not changed by 100 μM genistein treatment (data not shown). Under the same conditions, tyrosine phosphorylation of the above many proteins including the 145 kDa protein was inhibited by genistein (Fig. 2B).

A 42 kDa protein was also tyrosine phosphorylated by stimuli such as *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbor myristate acetate (PMA), whereas tyrosine phosphorylation of other proteins including 145 kDa protein was not induced by PMA and FMLP (data not shown).

### *3.2. Identification of a 145 kDa phosphotyrosine protein as the PLCγ 2*

It has been previously reported in human PMNs that upon Fcγ receptor activation tyrosine phosphorylation of PLCγ 2 with a molecular mass of 145 kDa occurred [7]. We tested the resting and pervanadate treated cell homogenates with an anti-PLCγ 2 antibody. The 145 kDa protein which was phosphorylated by pervanadate treatment of PMNs was found to be identical with the protein recognized by this

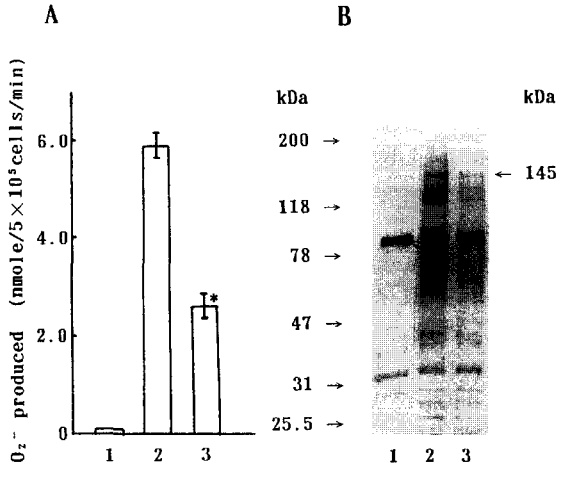


Fig. 2. Effect of genistein-pretreatment on $O_2^-$ production and tyrosine phosphorylation of proteins of PMNs by pervanadate treatment. PMNs ($10^7$ cells/ml) were preincubated in the presence and absence of genistein (100 μM) in KRP for 5 min at 37°C, and then further incubated with pervanadate (100 μM) for 3 min at 37°C. Then, the cell suspensions were withdrawn and analyzed for $O_2^-$ production (A) and phosphotyrosine proteins (B) as described in Section 2. Bars show the S.E. ($n = 6$). Numbers below the columns show the samples of PMNs without genistein and pervanadate (resting PMNs)(1), PMNs with pervanadate but without genistein (2), and PMNs with both genistein and pervanadate (3). * Significantly different from that of non-treated PMNs with genistein ($P < 0.01$).

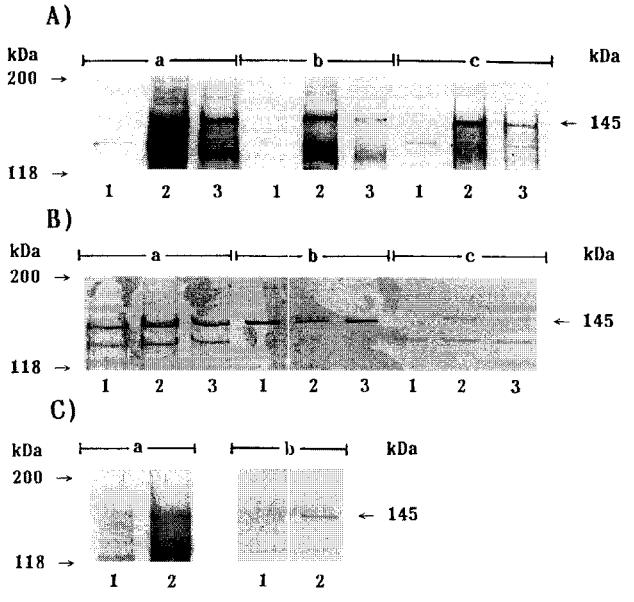


Fig. 3. Immunodetection of phosphotyrosine proteins and PLCγ 2 using specific antibodies. PMNs ($10^7$ cells/ml) were preincubated in the presence and absence of genistein (100 μM) in KRP at 37°C for 5 min, and then further incubated with or without pervanadate (100 μM) for 3 min. After incubation, cells were separated, resuspended in the extraction buffer, and sonicated to obtain whole extracts. From the whole extract of the above cell preparations, the cytosol and membrane fractions were prepared, and each fraction and whole extract in lysis buffer was subjected to Western blot analysis, as described in Section 2. Mouse anti-phosphotyrosine antibody and rabbit anti-PLCγ 2 antibody were used to detect the phosphotyrosine proteins and PLCγ 2, respectively. (A and B) Whole extract (a), cytosol fraction (b), and membrane (c) fractions from resting (lane 1), pervanadate-treated (lane 2), and genistein-pretreated then pervanadate-treated (lane 3) PMNs have been subjected to electrophoresis and immunoblotted with anti-phosphotyrosine antibody (A) or with anti-PLCγ 2 antibody (B) as described in Section 2. (C) Materials immunoprecipitated with anti-phosphotyrosine antibody from cytosol fraction of resting (lane 1) and pervanadate-treated (lane 2) PMNs, were subjected to electrophoresis, electroblotted and probed with anti-phosphotyrosine antibody (a) or anti-PLCγ 2 antibody (b) as described in Section 2.

anti-PLCγ 2 antibody (lane 2 of Fig. 3A and B). To confirm the identity of this protein as PLCγ 2, cytosol fraction from resting and pervanadate treated PMNs were immunoprecipitated with anti-phosphotyrosine antibody conjugated with agarose, and then immunoblotted with the same anti-phosphotyrosine antibody (Fig. 3C-a) or with anti-PLCγ 2 antibody (Fig. 3C-b). Anti-phosphotyrosine antibody precipitated from the cytosol fraction of pervanadate treated PMNs a 145 kDa protein. The protein was found to be positive on blots by both anti-phosphotyrosine antibody and anti-PLCγ 2 antibody. The above findings were not observed with the cytosol fraction of the resting cells. These findings strongly suggest that one of the 145 kDa phosphoproteins that appeared during respiratory burst of PMNs by pervanadate treatment is the PLCγ 2.

### *3.3. Translocation of PLCγ 2 in pervanadate treated PMNs*

To examine the distribution of PLCγ 2 in guinea pig PMNs, Western blotting using anti-PLCγ 2 and anti-phosphotyrosine antibodies was performed on cytosol and membrane fractions obtained from resting, pervanadate treated, and genistein-pretreatment followed by pervanadate treated PMNs. As shown in lane 1 of Fig. 3B-b and -c, PLCγ 2 was detected in the cytosol fraction of resting PMNs, but not in the membrane fraction. However, after treatment of PMNs with pervanadate, PLCγ 2 was detected in the membrane fraction (lane 2 of Fig. 3B-c), and the amount of PLCγ 2 in the cytosol fraction of the pervanadate treated cells was less than that of resting cells (lanes 1 and 2 of Fig. 3B-b). A 145 kDa phosphotyrosine protein was detected in both cytosol and membrane fractions of pervanadate treated PMNs (lane 2 of Fig. 3A-b and -c). In addition, by the pretreatment of PMNs with genistein, the amount of tyrosine phosphorylated 145 kDa protein and PLCγ 2 in the membrane fractions was lower than that in the membrane fraction of PMNs without genistein-pretreatment (lanes 2 and 3 of Fig. 3A-c and Fig. 3B-c). These findings suggest that PLCγ 2 exists in the cytosol fraction of resting cells and upon phosphorylation of PLCγ 2 translocation of the phosphorylated enzyme from cytosol to membrane occurs. We also carried out Western blots using the anti-PLCγ 1 antibody on the cytosol and membrane fractions of both resting and pervanadate treated PMNs. PLCγ 1 was also detected only in the cytosol fraction of resting cells, but much less than the amount of PLCγ 2. With PLCγ 1, we could not detect the translocation upon pervanadate treatment (data not shown).

### 3.4. PLC activity of pervanadate treated PMNs

Upon pervanadate treatment of guinea pig PMNs as described above, phosphorylation of PLCγ 2 was caused and then translocation of the phosphorylated enzyme from the cytosol to membrane occurred. Phosphorylation of PLCγ 2 in human PMNs and HL 60 cells results in promotion of phosphatidylinositide turnover [6,7]. Thus, we determined the total PLC activity of resting and pervanadate treated PMNs.

As shown in Table 1, total PLC activity was found to be increased by pervanadate treatment of PMNs, and this increase was reduced by pretreatment of PMNs with genistein. Then, we compared the total PLC activity of the cytosol and membrane fractions obtained from the above three types of PMNs, i.e., resting, pervanadate treated, and genistein-pretreatment followed by pervanadate treated cells (Table 1). PLC activity of the cytosol fraction was reduced largely by pervanadate treatment, and this reduction was inhibited partly by pretreatment of PMNs with genistein. On the other hand, PLC activity of membrane fraction was increased significantly by pervanadate treatment, and this increase was inhibited by pretreatment of PMNs with genistein. The above observed distribution of PLC activity in the cytosol and membrane fractions of the three types of cells was similar to that of PLCγ 2 (Fig. 3B-b and -c). These findings strongly support the view that PLCγ 2 in the cytosol is transferred to the membrane from cytosol through tyrosine phosphorylation of the enzyme.

## 4. Discussion

In the present experiments, we examined in detail the tyrosine phosphorylation of proteins, especially PLCγ 2, during activation of respiratory burst of guinea pig PMNs. When PMNs were incubated with pervanadate, activation of NADPH oxidase was observed at the stage that tyrosine phosphorylation of many proteins (145, 120, 104, 76, 68, 60, 53, 42, 37, 28, and 25 kDa) occurred rapidly. Tyrosine phosphorylation of these proteins was largely inhibited by pretreatment of PMNs with genistein, a tyrosine kinase inhibitor. Under the same conditions, activation of NADPH oxidase of PMNs was similary depressed significantly. It is obscure at present which protein(s) is actually involved in activation of NADPH oxidase. Among the proteins that tyrosine phosphorylation was caused by pervanadate treatment of PMNs and

Table 1
PLC activity of whole extract, cytosol fraction and membrane fraction

| | PLC activity (nmole/min/mg protein) | | |
|---|---|---|---|
| | None[a] | Pervanadate[b] | Genistein + pervanadate[c] |
| Whole extract | 12.744 ± 0.628 | 15.236 ± 0.828 (d) | 13.000 ± 0.557 (e) |
| Cytosol fraction | 11.357 ± 0.471 | 6.808 ± 0.339 (f) | 9.145 ± 0.535 (g) |
| Membrane fraction | 4.305 ± 0.729 | 5.967 ± 0.732 (h) | 4.733 ± 0.839 (i) |

PMNs ($10^7$ cells/ml) were pretreated with and without genistein (100 μM) for 5 min at 37°C, and then stimulated with pervanadate (100 μM) for 3 min at 37°C. After incubation, cells were separated, resuspended, and sonicated. The sonicated solution was used as the whole extract. Preparation of the cytosol and the membrane fractions from the above whole extract and assay for PLC activity were as described in Section 2. Data are expressed as mean ± S.E. of four to twelve experiments.

[a] None: resting PMNs, [b] Pervanadate: pervanadate-stimulated PMNs, [c] Genistein + pervanadate: PMNs pretreated with genistein before pervanadate stimulation.

Significantly different: d, $P < 0.05$; e, $P < 0.05$; f, $P < 0.001$; g, $P < 0.005$; h, $P < 0.001$; and i, $P < 0.01$.

its phosphorylation was inhibited by genistein, a 145 kDa protein was identified as PLCγ 2 on Western blots using anti-PLCγ 2 antibody, and PLCγ 2 was found to be expressed as a major component of the PLCγ family in the cytosol fraction of resting cells. There is a possibility that the 145 kDa protein phosphorylated under our experimental conditions is not only PLCγ 2, as other tyrosine phosphorylated proteins of this size unrelated to PLCγ 2 appear in stimulated neutrophils [15,16]. Upon treatment of PMNs with pervanadate, tyrosine phosphorylation of PLCγ 2 was induced and the phosphorylated enzyme was translocated from the cytosol fraction to the membrane fraction. The translocation of PLCγ 2 was inhibited by pretreatment of PMNs with genistein. Furthermore, PLC activity of pervanadate treated PMNs was higher than that of resting cells. Comparison of the PLC activity of cytosol and membrane fractions obtained from resting PMNs with that of pervanadate treated PMNs revealed that the enzyme activity of cytosol fraction from the latter cells was significantly lower than that from the former cells, whereas that of the membrane fraction obtained from the latter cells was significantly higher than that from the former cells. Only a fraction of the PLC activity that disappeared from the cytosol was recovered in the membrane, suggesting that some PLC in the cytosol might be inactivated by $H_2O_2$ or $O_2^-$ generated in pervanadate treated PMNs. The change of PLC activity in the cytosol and membrane fractions was similar to the change of PLCγ 2 protein levels (Fig. 3B-b and -c). These findings strongly support the view that PLCγ 2 is translocated from the cytosol to membrane through tyrosine phosphorylation of the enzyme. These findings suggest that upon treatment of PMNs with pervanadate PLCγ 2 present in the cytosol is phosphorylated at tyrosine residue(s) and then associates to the membrane fraction, resulting in a stimulation of second messanger production such as diglyceride and inositol phosphates from the plasma membrane.

Dusi et al. recently reported that in human PMNs upon Fcγ receptor activation by insoluble immune complex PLCγ 2 is phosphorylated at the tyrosine residue and phosphoinositide hydrolysis is stimulated [7]. Bianchini et al. have also reported that in HL 60 cells vanadate in the presence of $H_2O_2$ promotes the PLCγ 2-mediated production of $IP_3$ through tyrosine phosphorylation of PLCγ 2 [6]. In addition, Todderud et al. reported that epidermal growth factor treatment of A-431 epidermoid carcinoma cells elicits a redistribution of PLCγ 1 from a predominantly cytosol localization to membrane fractions, and that the amount of PLCγ 1 associated with membrane fractions coincides with the stoichimetry of growth factor-induced tyrosine phosphorylation of PLCγ 1 [17]. Therefore, our findings may suggest that the membranous association of PLCγ 2 is a significant event in signal transduction in PMNs functions. However, the exact physiological role of PLCγ 2 in respiratory burst of PMNs remains obscure.

The PLCβ family is regulated by G-proteins [18,19], while the PLCγ family is regulated through tyrosine phosphorylation by tyrosine kinase [20–22]. It is generally accepted that binding of agonist to their receptors stimulates PLC, possibly PLCβ family, through the activation of G-proteins in PMNs [4]. The PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into 1,2-diacylglycerol, a PKC activator, and $IP_3$, a calcium ion mobilizer. The activation of PKC leads to activation of NADPH oxidase. FMLP stimulates PMNs to activate NADPH oxidase through the receptor mediated signaling pathways [23], while PMA activates directly the respiratory burst in PMNs [24,25]. However, PKC inhibitors failed to inhibit most of the responses of PMNs by FMLP [26], and the presence of distinct pathways for FMLP- and PMA-induced $O_2^-$ generation of PMNs has been suggested [27]. We also observed that $O_2^-$ production ofPMNs by pervanadate treatment was not affected by the pretreatment of PMNs with staurosporine, even at sufficient concentrations of staurosporine which can inhibit significantly $O_2^-$ production by PMA (unpublished data). Therefore, the activation routes of NADPH oxidase by pervanadate treatment of PMNs may be a PKC- independent pathway through phosphorylation of PLCγ 2.

The present study showed that tyrosine residue(s) of PLCγ 2 was phosphorylated and the isozyme was translocated from cytosol to membrane in PMNs by pervanadate treatment. This is the first report on such phenomena with PMNs. However, further investigations are required to clarify precisely where tyrosine-phosphorylated and translocated PLCγ 2 is involved in the activation of NADPH oxidase and to elucidate the role of the tyrosine phosphatase, which is inhib-

ited by vanadate and dephosphorylates phosphorylation of tyrosine residues of PLCγ 2.

## Acknowledgements

This work was supported by grants from Japan Health Sciences Foundation and the Ministry of Welfare of Japan, and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

## References

[1] Goldstein, I.M., Roos, D., Kaplan, H.B. and Weissmann, G. (1975) J. Clin. Invest. 56, 1155–1163.
[2] Rossi, F. (1986) Biochim. Biophys. Acta 56, 1155–1163.
[3] Babior, B.M. (1978) N. Eng. J. Med. 298, 659–668.
[4] Cockcroft, S. and Gomperts, B.D. (1986) Nature 314, 534–536.
[5] Tauber, A.I. (1987) Blood 69, 711–720.
[6] Bianchini, L., Todderud, G. and Grinstein, S. (1993) J. Biol. Chem. 268, 3357–3363.
[7] Dusi, S., Donini, M., Bianca, V.D. and Rossi, F. (1994) Biochem. Biophys. Res. Commun. 201, 1100–1108.
[8] O'Shea, J.J., McVicar, D.W., Bailey, T.L., Burns, C. and Smyth, M.J. (1992) Proc. Natl. Acad. Sci. USA 89, 10306–10310.
[9] Teshima, R., Ikebuchi, H., Nakanishi, M. and Sawada, J. (1994) Biochem. J. 302, 867–874.
[10] Sbarra, A.J. and Karnovsky, M.L. (1959) J. Biol. Chem. 234, 1355–1362.
[11] Market, M., Andrews, P.C. and Babior, B.M. (1984) Methods Enzymol. 105, 358
[12] Shimohama, S., Fujimoto, S., Taniguchi, T. and Kimura, J. (1992) Brain Res. 579, 347–349.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[15] Liu, L., Damen, J.E., Cutler, R.L. and Krystal, G. (1994) Mol. Cell. Biol. 14, 6926–6935.
[16] Ptasznik, A., Traynor-Kaplan, A. and Bokoch, G.M. (1995) J. Biol. Chem. 270, 19969–19973.
[17] Todderud, G., Wahl, M.I., Rhee, S.G. and Carpenter, G. (1990) Science 249, 296–298.
[18] Smrcka, A.V., Hepler, J.R., Brown, K.O. and Sternweis, P.C. (1991) Science 251, 804–807.
[19] Taylor, S.J., Chae, H.Z., Rhee, S.G. and Exton, J.H. (1991) Nature (London) 350, 516–518.
[20] Wahl, M.I., Nishibe, S., Suh, P.-G., Rhee, S.G. and Carpenter, G. (1989) Proc. Natl. Acad. Sci. USA 86, 1568–1572.
[21] Meinhelder, J., Suh, P.G., Rhee, S.-G. and Hunter, T. (1989) Cell 57, 1109–1122.
[22] Nishibe, S., Wahl, M.I., Hernandez-Sotmayor, S.M.T., Tonks, N.K., Rhee, S.G. and Carpenter, G. (1990) Science 250, 1253–1256.
[23] Cockcroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.
[24] Romeo, D., Jug, M., Zabucchi, G. and Rossi, F. (1974) FEBS Lett. 42, 90–93.
[25] Lad, P.M., Olson, C.V. and Grewal, I.S. (1985) Biochem. Biophys. Res. Commun. 129, 632–638.
[26] Sha'afi, R.I., Molski, T.F., Gomez-Cambronero, J. and Huang, C.K. (1988) J. Leukocyte Biol. 43, 18–27.
[27] Robinson, J.M., Heyworth, P.G. and Badwey, J.A. (1990) Biochim. Biophys. Acta 1055, 55–62.